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Growth and survival of blowfly *Lucilia sericata* larvae under simulated wound conditions: implications for maggot debridement therapy

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Abstract. Maggot debridement therapy has become a well-established method of wound debridement. Despite its success, little information is available about the optimum duration of the treatment cycle and larval growth in wounds. This study examines the development of *Lucilia sericata* (Diptera: Calliphoridae) larvae under two containment conditions (bagged and free range) under simulated wound conditions and assesses the impact of transport and further storage of larvae on their survival and growth. There was no significant difference in size between bagged and free-range larvae over the 72-h experimental period. Larvae grew fastest 8–24 h after inoculation and completed their growth at 40–48 h. Mortality rates were similar (0.12–0.23% per hour) in both containment conditions and did not differ significantly ($P = 0.3212$). Survival of free-range larvae was on average 16% lower than survival of bagged larvae. Refrigeration of larvae upon simulated delivery for > 1 day reduced their survival to < 50% and caused a reduction in growth of up to 30% at 12 h, but not at 48 h, of incubation. Therefore, it is recommended that free-range larvae are left in the wound for a maximum of 40–48 h, and bagged larvae for 48–72 h. Larvae should be used within 24 h of delivery to avoid high mortality caused by prolonged refrigeration.

Key words. Biosurgery, larval development, medicinal maggots, storage, surgical maggots.

Introduction

Maggot debridement therapy (MDT) is an effective method of wound debridement and has become an established remedy in the treatment of chronic non-healing wounds (Mumcuoglu *et al.*, 1999; Wolff & Hansson, 2003; Sherman, 2009; Opletalová *et al.*, 2012; Mudge *et al.*, 2014). During this treatment, larvae of necrophagous flies are applied to wounds to remove necrotic tissue. Live medical-grade larvae of the greenbottle fly *Lucilia sericata* (Meigen) are typically exploited for this purpose, although other fly species, such as *Lucilia cuprina* (Wiedemann), *Protophormia terraenovae* (Robineau-Desvoidy) (Diptera: Calliphoridae) and *Musca domestica* L. (Diptera: Muscidae), have been used in some cases (Nuesch *et al.*, 2002; Li *et al.*, 2009; Paul *et al.*, 2009; Tantawi *et al.*, 2010). *Lucilia sericata* larvae have been shown to produce an array of bioactive compounds that aid in wound healing, promote angiogenesis and tissue regeneration, reduce inflammation and eliminate bacteria (Nigam *et al.*, 2010; Telford *et al.*, 2011; Cazander *et al.*, 2013). Some of these compounds show promise as novel antibacterial agents (Cazander *et al.*, 2013). With the introduction of new dressings that contain the larvae more reliably, as well as new scientific evidence for the effects of substances secreted by the larvae, the popularity of larval therapy has increased substantially among clinicians and patients alike (Sherman, 2009).

Despite this popularity, however, there is a great deal of conflicting information about the recommended duration of treatment and the efficacy of using 'bagged' larvae. This is primarily

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the result of insufficient knowledge of larval development in wounds under the restrictive conditions of MDT. For example, in his review, Sherman (2009) notes that larvae are typically left in the wound for 48–72 h, after which they are satiated, have 'finished working', and therefore can be removed. By contrast, Blake *et al.* (2007) showed in laboratory experiments that the weight of larval substrate (porcine muscle) after 4 days of simulated maggot therapy was significantly lower than after 3 days and hence recommended 4-day application intervals for MDT. There is ongoing controversy around the use and effectiveness of bagged larvae, which are sealed in a fine net to allow more convenient application, the better containment of larvae and less movement of the dressing during the treatment (Grassberger & Fleischmann, 2002). It has not been clearly established whether the presence of the net bag interferes with larval activities and decreases the amount of necrotic tissue consumed by the larvae. Evidence in support of the superior debridement capabilities of free-range larvae in clinical practice (Steenvoorde *et al.*, 2005) and their faster growth under laboratory conditions (Thomas *et al.*, 2002) has been presented. However, others have shown that bagged larvae can be as efficient in wound debridement as their free-range counterparts in both laboratory and wound settings (Blake *et al.*, 2007; Dumville *et al.*, 2009).

Information on larval development in wounds is crucial to determine the optimum duration of larval therapy with respect to the potential benefits (debridement, release of digestive enzymes and other bioactive substances) delivered by the larvae. The research literature to date has assessed the use and effectiveness of bagged larvae, but has focused on the determination of the treatment outcome under different types of containment (Steenvoorde *et al.*, 2005; Blake *et al.*, 2007; Dumville *et al.*, 2009) or investigated the growth of larvae under conditions that cannot be considered representative of the wound environment, such as when larvae are incubated at room temperature or fed with fetal bovine serum as model wound fluid (Thomas *et al.*, 2002).

Another practical problem associated with larval therapy concerns the short-term storage of larvae upon delivery in situations in which medical staff need to postpone their application (for instance, because there is a rapid change in the condition of the wound or because the patient reschedules a planned appointment at an outpatient clinic). The larvae used for MDT are typically supplied by external organizations and delivered to health care facilities by overnight courier services (Sherman, 2009), although some hospitals keep their own fly colonies to ensure easy access to sterile larvae (Wolff & Hansson, 2005). It is recommended that because young larvae, which need access to sufficient food, water and oxygen, have a high likelihood of mortality, they should be applied to the wound no later than 24 h after delivery (Sherman, 2009). Rosales *et al.* (2004) also reported a very short shelf life of larvae but suggested that the refrigeration of larvae at 5.6 °C for up to 60 h prior to their placement on the wound may maintain favourable levels of larval motility. However, the effects of such storage on the viability and development of larvae remain unknown.

The present study was undertaken to investigate the growth and development of *L. sericata* larvae under simulated wound conditions and to examine in detail growth curves and survival of larvae under both bagged containment and free-range conditions. This study also sought to answer questions about the possible short-term storage of larvae destined for MDT, particularly in relation to the issues of larval survival of short-term refrigeration and how it might affect the further development of larvae, which are relevant to practitioners of MDT.

Materials and methods

Maintenance of flies

Lucilia sericata flies were maintained in the laboratories of Scientica s.r.o. in St Michael's Hospital, Bratislava, Slovakia, in 50.0 × 50.0 × 50.0-cm steel-frame cages covered with tulle netting (RNDr. Ondrej Šauša, Bratislava, Slovakia) at standard conditions [26 ± 2 °C, 40–60% relative humidity (RH), LD 12 : 12 h] with water and food (yeast hydrolysate and sugar cubes) provided *ad libitum*. To induce oviposition, a mixture of beef liver and wheat bran was provided in a Petri dish placed at the bottom of the cage.

Preparation of larvae for experiments

Larvae were prepared according to the protocol of a local supplier of sterile medical-grade larvae within Slovakia (MEDALT n.o., Bratislava, Slovakia; personal communication, 2012). Eggs were collected as described above and disinfected in a 0.25% chloramine solution as described by Wolff & Hansson (2005). Because this disinfection procedure is known to be reliable with rare instances of bacterial contamination of the disinfected eggs [Wolff & Hansson, 2005; MEDALT n.o., personal communication, 2012 (effectiveness was also verified in preliminary experiments)], and in view of the large numbers of bagged and free-range larvae to be prepared for the experiments and given that the larvae were not destined for actual wound treatment, no follow-up tests to confirm the sterility of larvae were performed. Disinfected eggs were aseptically transferred to sterile media made by mixing heat-sterilized egg yolk powder with sterile distilled water. Petri dishes with fly eggs seeded on egg yolk media were incubated at 28 ± 2 °C overnight. Incubation at a higher temperature (28 °C) rather than standard fly rearing temperature (26 °C) was used to speed up the hatching of eggs and to increase the feeding time of newly hatched larvae in order to improve their survival during the period of transport, in which they have no access to food. The next morning, newly hatched first instars were washed from the lids of Petri dishes with sterile distilled water. A total of 125 larvae were hand-counted aseptically with sterile tweezers and were either placed in a sterile flask together with 0.1 mL of sterile distilled water ('free-range larvae') (Figure S1A) or sealed in a sterile 5.0 × 5.0-cm polyamide bag (mesh size: 99 μm) with a cotton cloth measuring 2.5 × 2.5 cm, moistened with 0.5 mL of sterile distilled water, and placed in a sterile Petri dish (10.0 cm in diameter) secured with sticky tape ('bagged larvae') (Figure S1B). Different volumes of water were added to the flasks and bags to ensure optimal hydration and survival of the larvae. The number of larvae was chosen to correspond to five larvae/cm^2 of folded bag surface,

which was previously used to estimate the growth of larvae in wounds (Čičková *et al.*, 2013). Flasks and Petri dishes were placed in a transportation box measuring 23.0 × 20.5 × 25.0 cm (AcuTemp Thermal Systems, Moraine, OH, U.S.A.) together with six cooling pads (ClimSel C 7N; Climator Sweden AB, Skövde, Sweden) previously cooled to 5–6 °C, and subjected to simulated transport for 24 h. With cooling pads, the temperature inside the transport box was maintained at 9–17 °C (Figure S2). This transport protocol reflects conditions typical of those under which larvae are distributed within Slovakia by a local manufacturer (MEDALT n.o., personal communication, 2012).

Larval growth and development under simulated wound conditions

Nine bags and nine flasks of larvae were subjected to simulated transport conditions in the transportation box. After 24 h, the free-range or bagged larvae were transferred to a slice of pork measuring approximately 5.0 × 5.0 cm (approximately 20 g; non-sterile boneless lean loin cuts, intended for human consumption) in a small aluminium feeding box covered with two squares of sterile gauze (5.0 × 5.0 cm) and incubated in a net-covered plastic box in an incubator at 35 ± 2 °C, RH 70–90% in the dark for 8, 16, 24, 32, 40, 48, 56, 64 and 72 h. Liver is typically used in the laboratory rearing of *L. sericata* larvae; however, it was not suitable in the present experimental work because at 35 °C liver releases large amounts of liquid, which resulted in the drowning of bagged larvae (despite drainage with dry gauze) and the escape of free-range larvae from the feeding box.

Larvae were checked every 8 h and gauze squares were replaced if they became fully soaked with larval excretions. Following the incubation on meat, the bags of larvae were cut open (free-range larvae were picked up with tweezers) and larvae from each replicate were washed with tap water, placed in labelled Petri dishes and freeze-killed at −20 °C. Although it is recommended that larvae are killed using boiling water when sizes are to be measured (Adams & Hall, 2003), the present group chose to freeze the larvae in order to ensure that the resulting data were comparable with those of a previous study of larval growth in wounds in which larvae were freeze-killed in order to reduce the risk of escape of infectious larvae (Čičková *et al.*, 2013). Maximum length and width, as well as the larval instar of surviving larvae within the replicate, were then measured under a dissecting microscope to the nearest 0.1 mm. For each replicate, the average length and width of surviving larvae, and the proportions of first, second and third instars, and larval survival at each time-point (8, 16, 24, 32, 40, 48, 56, 64 and 72 h of incubation) were recorded. All treatments were replicated five times except those for bagged larvae at 8 h and free-range larvae at 8, 16, and 24 h, which were replicated four times.

On two separate occasions, additional samples of larvae in flasks were exposed to simulated transport conditions before the larvae were freeze-killed and measured to assess their size at the beginning of the experiment (i.e. at 0 h).

Larval survival and growth after storage for prolonged time periods

Ten flasks and 10 bags of larvae were prepared as described above and subjected to the following treatments: transport (with cooling pads maintaining a temperature of 9–17 °C) for 24 h; transport followed by storage in a refrigerator at 7–8 °C for 1, 2, 3, 4, 5, 6 and 7 days, respectively, and exposure to ambient temperature (24 ± 2 °C) for 24 and 48 h, respectively (equivalent to transport without cooling pads). Immediately after the treatments, the bags were cut open or the larvae were washed from the flasks. Numbers of live and dead larvae were counted. The experiment was replicated 10 times.

To assess the potential negative effects of storage on larval growth and development, larvae were also examined after incubation on pork under simulated wound conditions. Eleven flasks and bags of larvae were prepared as described above and subjected to no treatment (control), transport (with cooling at 9–17 °C) for 24 h, transport followed by storage in a refrigerator at 7–8 °C for 1, 2, 3, 4, 5, 6 and 7 days, respectively, and incubation at ambient temperature (24 ± 2 °C) for 24 and 48 h, respectively. Following the treatments, the free-range larvae or bags were placed on a slice of pork measuring approximately 5.0 × 5.0 cm (approximately 20 g) in a small aluminium box, covered with two squares of sterile gauze (5.0 × 5.0 cm), and incubated in a net-covered plastic box at 35 ± 2 °C, RH 70–90% in the dark for 12 and 48 h, respectively. Larvae were checked every 8–12 h, and gauze soaked with maggot excretions was replaced if necessary. Following this incubation on meat, the bags of larvae were cut open (free-range larvae were picked up with tweezers), and larvae were washed with tap water, placed in labelled Petri dishes and freeze-killed at −20 °C. All treatments were replicated five times, except those for bagged larvae refrigerated for 1 and 3 days and incubated for 12 h, bagged larvae stored for 0 h (control) and refrigerated for 3 days and incubated for 48 h, and all storage treatments of free-range maggots that were incubated for 12 h, which were replicated four times. Maximum lengths and widths were then measured under a dissecting microscope to the nearest 0.1 mm. For each replicate, the average length and width of the surviving larvae and larval survival were determined.

Statistical analysis

Prior to analyses, the data were checked for normality and homoscedasticity using the Shapiro–Wilk test and Bartlett's test, respectively. If necessary, common data transformations were employed to satisfy the assumptions of parametric statistical tests. All analyses were performed in R Version 3.0.0 (R Core Team, 2013).

Larval growth and development. Square root-transformed larval lengths and log-transformed larval widths were analysed by two-way analysis of variance (ANOVA) for unbalanced designs [type III sum of squares (Fox *et al.*, 2013)] with time and containment type (free-range or bagged) as fixed-effect

experimental factors, followed by the Tukey–Kramer procedure to identify significant differences between the means (Zar, 2010). Survival data (separately for free-range and bagged larvae) were subjected to simple linear regression; after confirming the linearity of the regression, the regression slopes were compared by Welch's *t*-test (Zar, 2010). Proportions of first, second and third instars exhibited large departures from normality and homoscedasticity and failed to meet the assumptions of parametric and non-parametric tests even after common data transformations, and therefore were not compared statistically. A special Microsoft Excel template created by Weissgerber *et al.* (2015) was used to plot larval instar data in Fig. 1.

Larval survival and growth after storage for prolonged time periods. Data on the survival of larvae immediately after different storage treatments did not conform to the assumptions of parametric and non-parametric analyses, even after data transformations, and thus were not compared statistically. Arcsine-transformed survival and untransformed lengths and widths of larvae following storage and incubation at 35 °C for 12 and 48 h, respectively, were analysed by two-way ANOVA for unbalanced designs (type III SS) with storage and containment as fixed-effect factors. The Tukey–Kramer test was used to separate significantly different means as appropriate. Because of issues of variability in survival data (zero variance after prolonged storage) and missing measurements of length and width caused by 100% larval mortality, results of storage following transport with 4–7 days of refrigeration and 2 days at 24 °C were omitted from the datasets used for statistical analyses, although the data are still shown in tables and charts.

Results

Larval growth and development

At the time of inoculation, the larvae measured a mean ± standard error of the mean (SEM) of 2.65 ± 0.03 mm in length and 0.50 ± 0.01 mm in width and consisted of 14.9 ± 4.9% first and 85.1 ± 4.9% second instars. The larvae grew very little during the first 8 h of incubation. The fastest growth was observed between 8 and 24 h of the experiment (Fig. 2). No significant differences in size between free-range and bagged larvae of the same age were observed. Statistical analysis of maggot lengths and widths showed significant effects of time on larval size, but the effect of containment on either length (time: $F = 45.0875$, d.f. = 8,68; $P < 2 \times 10^{-16}$; containment: $F = 0.9729$, d.f. = 1,68; $P = 0.3275$; interaction: $F = 1.6608$, d.f. = 8,68; $P = 0.1242$) or width (time: $F = 84.2078$, d.f. = 8,68; $P < 2 \times 10^{-16}$; containment: $F = 1.7142$, d.f. = 1,68; $P = 0.1948$; interaction: $F = 1.8114$, d.f. = 8,68; $P = 0.0899$) was not significant. Greater variability between replicates in the average size of bagged larvae was observed towards the end of the experiment. In three of five bags incubated for 56 h and two of five bags incubated for 64 h, larvae grew more slowly (resulting in a rather low but not significant *P*-value for interaction of time and containment type on maggot growth), although ample food sources were available.

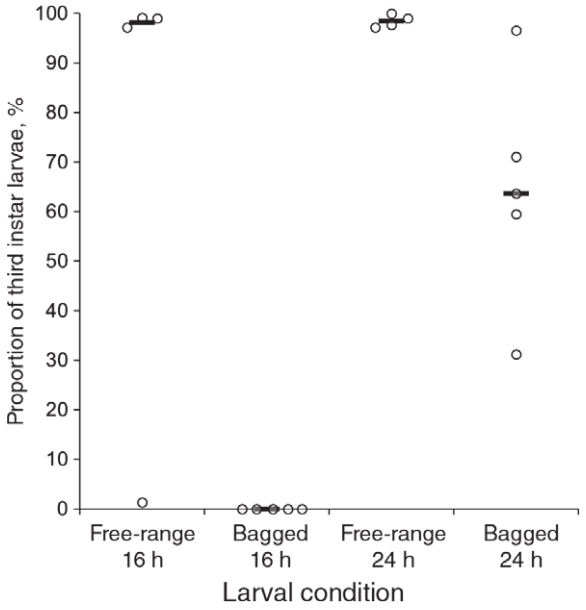

Fig. 1. Timing of the second moult of bagged and free-range *Lucilia sericata* larvae during development under simulated wound conditions. Open circles indicate individual observations of the proportions of third instar larvae among all survivors; black horizontal bars indicate group medians. Replicates: $n = 4$ for free-range larvae; $n = 5$ for bagged larvae.

The timing of the first moult was similar in both groups of larvae, but the second moulting occurred earlier in free-range larvae. A total of 98.4% of free-range larvae reached the third instar after 24 h of incubation at 35 °C, compared with only 64.4% of their bagged counterparts (Fig. 1).

Visual inspection of the growth data (Fig. 2; Table S1), supported by the results of multiple comparison procedures, indicates that both free-range and bagged larvae completed their growth after 40–48 h of incubation. In the free-range maggot treatment, the first wandering larvae were observed as early as 40 h after inoculation; in the longer incubation treatments (> 40 h), the majority of larvae left the aluminium box and very few larvae remained feeding on meat.

The survival of larvae differed markedly between the bagged and free-range maggot groups. The number of surviving larvae in bags decreased linearly from 91.6% at 8 h to 74.9% at 72 h of incubation (Fig. 3). The survival of free-range larvae was very variable and, on average, 16% lower than that of bagged larvae. Linear regression lines fitted to the survival data gave a highly significant relationship for bagged larvae [$S_B = 94.337\ (\pm 2.401) - 0.230\ (\pm 0.053) \times h$, where S_B is the survival of bagged larvae at h hours from inoculation; regression slope: $t = -4.363$, d.f. = 42; $P < 0.0001$; $R^2 = 0.312$], but the regression coefficient (slope) for the survival of free-range larvae did not differ significantly from 0 [$S_F = 74.111\ (\pm 5.611) - 0.121\ (\pm 0.121) \times h$, where S_F is survival of free-range larvae at h hours from inoculation; regression slope: $t = -0.996$, d.f. = 40; $P = 0.3250$; $R^2 = 0.024$].

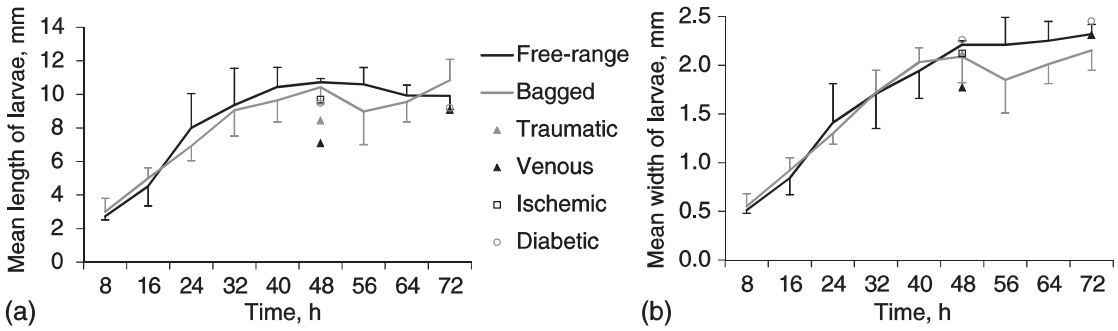

Fig. 2. Length (a) and width (b) of larvae under simulated wound conditions. Data are detransformed means of five replicates per treatment except for those with bagged larvae at 8 h and free-range larvae at 8, 16 and 24 h, which were replicated four times Error bars indicate 95% confidence intervals (only single sides of confidence intervals are shown for clarity). For comparison, data points showing the sizes of bagged larvae developing in traumatic, ischaemic, venous and diabetic wounds are plotted (data from Čičková *et al.*, 2013).

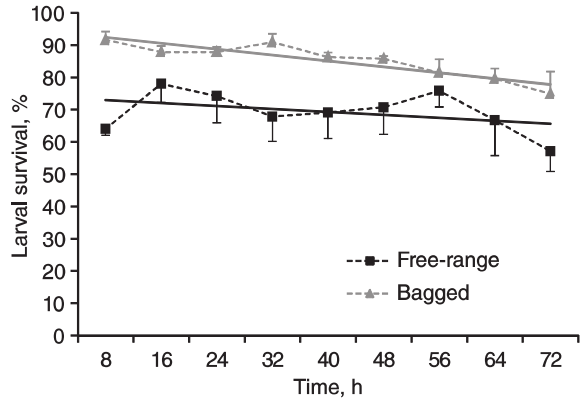

Fig. 3. Survival of larvae under simulated wound conditions. Solid lines correspond to regression equations obtained for each containment type. Error bars indicate 1 standard error of the mean (SEM). Each treatment was replicated five times except for those with bagged larvae at 8 h and free-range larvae at 8, 16 and 24 h, which were replicated four times.

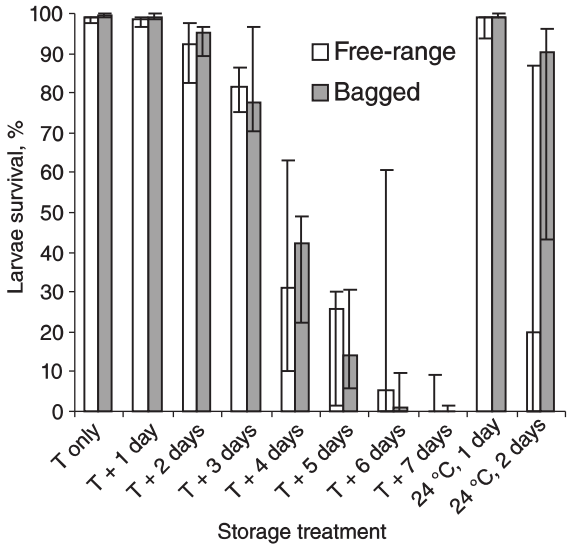

Fig. 4. Proportions of larvae surviving immediately after various storage treatments: transport only (simulated transport conditions for 24 h, with cooling pads); t + 1 days … t + 7 days, transport (with cooling pads) followed by 1–7 days of storage in a refrigerator at 7–8 °C; 24 °C 1 and 2 days, storage at ambient temperature (24 ± 2 °C) for 24 and 48 h, respectively. Data are medians of 10 replicates per treatment; error bars indicate interquartile ranges. T, transport.

Larval survival and growth after storage for prolonged periods

The survival of larvae immediately after simulated transport conditions (with cooling), after transport followed by 1 day of refrigeration and after 1 day at 24 °C, was consistently high (> 92% in all cases) in both the bagged and free-range maggot groups. Prolonged storage, with or without refrigeration, resulted in gradual increases in mortality and in the overall variability of results (Fig. 4). Additionally, decreased motility of larvae in both groups was observed after longer periods of storage in the refrigerator.

The survival of larvae exposed to the various storage treatments was further reduced after incubation under simulated wound conditions (Table 1). The highest rates of larval survival at 12 and 48 h were observed when no storage treatment was in place (i.e. in the control sample). Following simulated transport (with cooling), the number of surviving larvae remained relatively high (70 and 75% in free-range and bagged larvae, respectively, at 48 h). Further storage of larvae in the refrigerator caused a rapid increase in mortality, with significant differences between the two containment variants at 12 h ($F = 7.674$, d.f. = 1,40; $P = 0.0085$), but not at 48 h ($F = 1.146$, d.f. = 1,47; $P = 0.2899$) (Table 1). Storage of larvae at an ambient temperature (24 °C) for 1 day did not affect larval survival, but 2-day storage was lethal for most larvae.

Storage of the larvae resulted in significant differences in the size of larvae at 12 h of incubation (length: $F = 3.767$, d.f. = 5,37; $P = 0.0074$; width: $F = 2.964$, d.f. = 5,37; $P = 0.0239$) (Fig. 5), but not at 48 h of incubation (length: $F = 0.545$, d.f. = 5,43; $P = 0.7410$; width: $F = 1.705$, d.f. = 5,43; $P = 0.1541$). Moreover, differences in size between the two conditions of larval containment were observed. Bagged larvae were slightly, although not significantly ($F = 3.214$, d.f. = 1,37; $P = 0.0812$), longer than free-range larvae at 12 h, but free-range

Table 1. Survival of free-range and bagged larvae after short-term refrigeration followed by incubation in simulated wound conditions. Treatments were replicated five times.

	Survival, detransformed mean (95% CI)			
	12 h incubation		48 h incubation	
Storage treatment	Free-range larvae	Bagged larvae	Free-range larvae	Bagged larvae
Control	89.38%$*^{aA}$	98.41%^{aA}	86.21%^{a}	85.96%$*^{a}$
	(71.37–99.02)	(87.77–98.92)	(58.20–99.67)	(69.61–94.33)
Transport only	85.68%$*^{aA}$	93.01%^{abA}	70.50%^{ab}	75.44%^{a}
	(73.69–94.47)	(86.55–97.47)	(51.47–86.41)	(43.11–96.70)
Transport + 1 day	44.80%$*^{abA}$	83.13%^{abA}	39.69%$*^{bc}$	55.26%^{ab}
	(18.24–73.09)	(60.13–97.37)	(19.22–62.28)	(27.64–81.22)
Transport + 2 days	16.46%$*^{bA}$	67.25%^{bcB}	22.87%^{cd}	46.08%^{ab}
	(0.0–71.05)	(52.64–80.33)	(6.21–46.01)	(25.32–67.58)
Transport + 3 days	5.86%$*^{bA}$	31.41%^{cA}	2.56%$*^{d}$	15.19%$*^{b}$
	(0.0–52.78)	(1.43–76.95)	(0.0–25.14)	(1.98–47.08)
Transport + 4 days	0.00%$*$	17.06%	0.00%	8.17%
	(0.00–0.00)	(1.97–42.57)	(0.00–0.00)	(0.35–24.73)
Transport + 5 days	0.00%$*$	3.00%	0.00%	0.16%
	(0.00–0.00)	(0.00–25.89)	(0.00–0.00)	(0.00–2.30)
Transport + 6 days	0.43%$*$	0.03%	0.13%	0.26%
	(0.00–6.03)	(0.00–0.46)	(0.00–1.83)	(0.00–3.69)
Transport + 7 days	0.00%$*$	0.13%	0.00%	0.16%
	(0.00–0.00)	(0.00–0.93)	(0.00–0.00)	(0.00–2.30)
24 °C 1 day	77.73%$*^{aA}$	86.54%^{abA}	68.15%^{abc}	77.53%^{a}
	(65.39–87.99)	(62.27–99.20)	(28.12–96.52)	(62.32–89.69)
24 °C 2 days	0.00%$*$	32.00%	0.40%	43.18%
	(0.00–0.00)	(0.00–92.02)	(0.00–5.55)	(6.01–85.93)

$*$Treatments for which $n = 4$.
Means in columns followed by different superscript lowercase letters and means within rows followed by different superscript uppercase letters are significantly different at $P = 0.05$.
95% CI, 95% confidence interval.

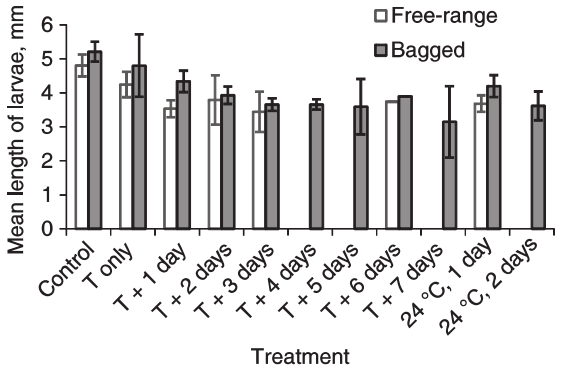

Fig. 5. Length of larvae subjected to different storage treatments after incubation at 35 °C for 12 h. Error bars indicate 1 standard error of the mean (SEM) (missing error bars represent single-observation values). T, transport.

larvae were significantly wider than bagged larvae at 48 h ($F = 8.008$, d.f. = 1,43; $P = 0.0070$).

Discussion

Information about the duration of treatment is essential for the success of MDT. Currently available data regarding the optimum duration of the MDT cycle and the effects of containment on the success of therapy are, however, conflicting. In their laboratory studies, Grassberger & Reiter (2001) showed that at 34 °C, a temperature close to that of human skin, the development of *L. sericata* from the egg to the prepupal 'wandering' stage on beef liver may take as little as 48 h. During MDT, however, young larvae are applied to wounds, which implies that the time until their development is completed is shorter. In this study, in which larvae were prepared in line with the protocols of a local supplier (MEDALT n.o.), the larvae intended for wound application consisted mostly of second instars and a small proportion of first instars. The present results suggest that free-range and bagged larvae may finish their growth under simulated wound conditions by 40–48 h. This is in agreement with Sherman (2009), who noted that the optimum duration of the MDT cycle should be 48–72 h, but contradicts the recommendation of Blake *et al.* (2007), who suggested that treatments with bagged or free-range larvae should be left to continue for up to 4 days because significantly more tissue was metabolized in 4 days than in 3 days. However, Blake *et al.* (2007) did not state which larval stage was applied to porcine muscle, and a slightly lower incubation temperature (32 °C) and different geographic origin of the larvae (Gallagher *et al.*, 2010) may cause some delay in larval development. Furthermore, Blake *et al.* (2007) did not directly assess larval growth, but instead measured the amount of metabolized larval

substrate. As the present data suggest that larval development will be completed by 48 h (and thus little to no material will be metabolized by the larvae beyond 48 h of their placement in the wound), it is also possible that some of the loss of substrate in the earlier study was caused by factors other than the larvae, such as bacterial decomposition or desiccation, which were not controlled for. However, further experimental work will be necessary to confirm these hypotheses.

The appearance of wandering larvae at > 40 h of incubation indicates that larvae no longer consume food at this time and instead concentrate their efforts on finding a suitable site for pupation. Additionally, wandering larvae no longer express lucifensin (Valachová *et al.*, 2013), a major antibacterial protein thought to contribute to the beneficial antimicrobial activity of maggot excretions/secretions (Čeřovský *et al.*, 2010). Leaving the larvae in the wound after they stop consuming necrotic tissue may have little benefit for the patient and may have unpleasant consequences if the larvae escape from the dressing.

The present data demonstrate that bagged larvae grow at a rate comparable with that of free-range larvae if they have access to an adequate food source, although some delay in the moulting of bagged larvae was observed. However, these findings disagree with those of Thomas *et al.* (2002), who found a highly significant difference in maggot growth, and numerically high (11%), but statistically insignificant ($P = 0.06$) difference in maggot survival during incubation on porcine liver in a dark moist environment. In their experiment, bagged larvae reached an average weight of 13 mg, but free-range larvae weighed 20 mg on average. Although a direct comparison with the present data on size is not possible, this discrepancy may be explained by the differences in experimental protocols. Clark *et al.* (2006) noted that the growth rates of *L. sericata* larvae on various body tissues may differ. Larvae fed beef or porcine liver were smaller and their development took longer than that of larvae fed lung or heart (Clark *et al.*, 2006), which may be indicative of the lower nutritional value of liver and suggests that the larvae used by Thomas *et al.* (2002) may have grown more slowly than the larvae used in the present study. The differences in environmental conditions (lower temperature, less nutritious substrate) in the study by Thomas *et al.* (2002) may have intensified any barrier effect of containment on bagged larvae. Although no difference in maggot size between the two containment types was observed in the current study, there was a delay of > 8 h in moulting, which suggests that the bag does have an influence on larval development.

The size and survival of bagged larvae at 48 and 72 h in the present study correspond well with findings in this group's previous research on the development of *L. sericata* in diabetic, ischaemic and traumatic wounds (Čičková *et al.*, 2013), except that bagged larvae used to debride venous ulcers for 48 h were smaller and suffered from greater mortality than bagged larvae of the same age in this study. This suggests that the present group's laboratory experiments with biobags may be used to reasonably approximate larval development in wounds, or may perhaps provide an optimistic estimate for some types of wound in which larvae do not grow so well. The fact that bagged larvae finished their growth after approximately 48 h of incubation under simulated wound conditions, but larvae in bags used for the treatment of venous ulcers continued to grow after 48 h and reached their final size at 72 h of MDT (Čičková *et al.*, 2013) indicates that a longer duration (72 h) of larval therapy with bagged larvae may be justified in some types of wound in which larvae grow more slowly. The use of sterilized (e.g. by high doses of γ-irradiation) meat in the experiments makes it possible to explore factors such as bacterial flora or medications that may be responsible for the reduced growth of larvae in some wounds.

The mortality rates observed in the present experiments are similar for bagged and free-range larvae. However, it is difficult to assess whether the mortality observed in free-range larvae corresponds to the actual mortality of larvae within wounds. It is experimentally difficult, if not impossible, to accurately simulate the dressing used in a classic MDT. One possible drawback of the present experimental set-up is that larvae in the free-range group were able to roam freely not only on the available food, but also on the walls of the aluminium feeding box and plastic box, and this may have contributed to the relatively high and very variable mortality observed in this study. Indeed, groups of young larvae on the inner walls of aluminium feeding boxes were routinely observed during regular 8-h checks, but no larvae were found outside the box until about 40 h of incubation, when larvae began to enter the wandering stage. Another possibility is that the transport conditions in the free-range larval group induced some kind of latent damage that did not result in immediate death (which would be seen as reduced numbers of surviving larvae upon removal from the refrigerator in Fig. 4), but manifested as increased mortality during subsequent larval development. Similar increases in larval mortality attributable to latent injury were demonstrated in refrigerated house fly (*Musca domestica* L.) eggs (Leopold, 2000; Čičková *et al.*, 2014). Blake *et al.* (2007) estimated median survival of 85 and 95% for bagged and free-range larvae, respectively, on the third day of their experiment. In the present study, median survival was lower (78.4 and 61.6% for bagged and free-range larvae, respectively, at 72 h), indicating that the experimental set-up and origin of the larvae may influence the mortality rates observed.

The second part of this study examined the possible storage of larvae prior to their application to the wound. The current results show that, once sterile larvae have been packed and sent to the clinic, they should be applied to the wound as soon as possible because prolonged starving and delayed application may result in low survival and often severe mortality rates. An unexpected and somewhat surprising finding was that larval survival after 1 day at an ambient temperature was comparable with that after storage for 1 day with cooling (transport-only treatment). This finding suggests that larvae may withstand the typical duration of transport ($\leq$ 24 h) without requiring a low-temperature environment to retain high survival rates. Apparently, the larvae accumulated enough nutrients during incubation on egg yolk media and the bag or flask contained sufficient moisture to sustain them for 24 h.

The refrigeration of larvae upon simulated delivery negatively affects early larval development, as evidenced by the reduced size of larvae at 12 h from inoculation. This may be the result of initially decreased maggot motility and the time needed to adjust to the new environmental conditions. However, as the size of larvae after 48 h of incubation did not reveal any significant effects of storage duration, the initial delay in growth is offset

in the later period of development. Interestingly, larval survival and growth appeared to be better in the bagged larvae treatment at 12 h. This may reflect the increased range of motion in the free-range larvae group. Bagged larvae, because they were contained in a bag, had a limited range of motion and were able to spend more time in contact with the food (albeit separated by the net).

The data presented in this study show that the containment of larvae in a bag during simulated MDT does not negatively impact their feeding and growth, although a delay in moulting indicates that the net may have a weak negative effect on their development. Upon delivery, bagged and free-range larvae may be stored in a refrigerator for 24 h. Longer refrigeration is not recommended because it has a serious negative impact on larval survival.

Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: DOI: 10.1111/mve.12135

Figure S1. (A) Free-range larvae in a flask and (B) bagged larvae, prepared for the experiment.

Figure S2. Environmental conditions during the simulated transport of larvae. Temperature and relative humidity were measured with an HOBO® logger on three separate occasions. Error bars indicate 1 standard error of the mean (SEM).

Table S1. Growth of larvae under simulated wound conditions.

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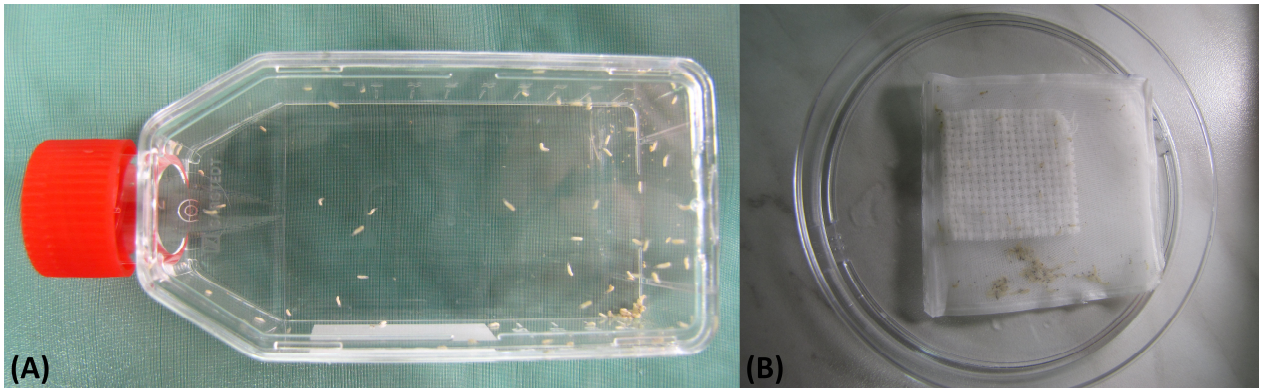

Fig. S1. Free-range maggots in a flask (A) and bagged maggots (B), prepared for the experiment.

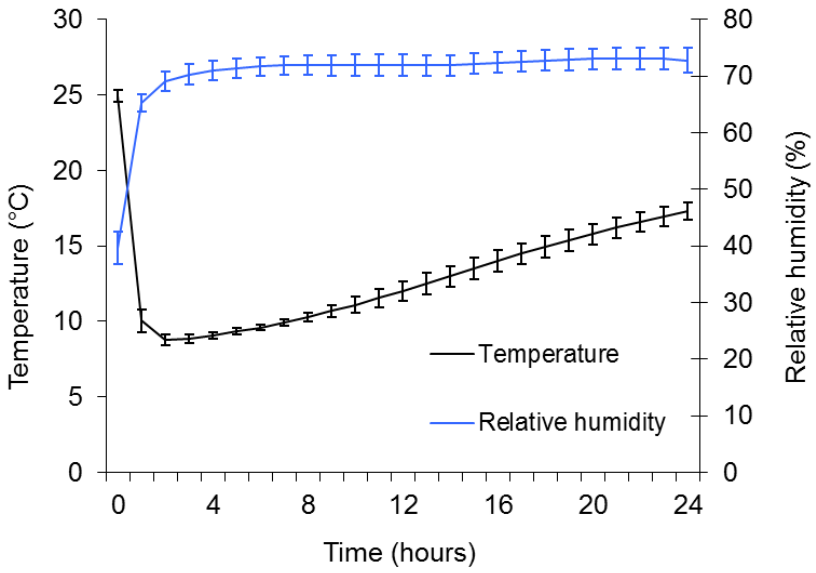

Fig. S2. Environmental conditions during simulated transport of the larvae. Temperature and relative humidity were measured with a HOBO® logger on three separate occasions. Error bars indicate 1 SEM.

Table S1. Growth of maggots under simulated wound conditions. Data are presented as detransformed means (95% confidence interval). *n*=5 per treatment except for bagged larvae at 8 h and free-range larvae at 8, 16, and 24 h, which were replicated 4 times

	Length (mm)		Width (mm)	
Time	free-range	bagged	free-range	bagged
8 h	2.73[a1]	2.99[a]	0.51[a]	0.55[a]
	(2.52-2.96)	(2.28-3.81)	(0.48-0.54)	(0.45-0.68)
16 h	4.53[b]	5.00[b]	0.84[b]	0.92[b]
	(3.35-5.89)	(4.41-5.63)	(0.67-1.05)	(0.81-1.05)
24 h	8.00[c]	6.91[c]	1.41[c]	1.30[c]
	(6.19-10.05)	(6.05-7.83)	(1.10-1.81)	(1.19-1.41)
32 h	9.35[cd]	9.05[d]	1.71[c]	1.72[d]
	(7.37-11.56)	(7.52-10.74)	(1.35-2.15)	(1.51-1.95)
40 h	10.44[d]	9.64[d]	1.94[cd]	2.03[d]
	(9.33-11.62)	(8.36-11.00)	(1.66-2.26)	(1.89-2.18)
48 h	10.73[d]	10.42[d]	2.21[d]	2.09[d]
	(10.50-10.95)	(9.44-11.45)	(2.17-2.25)	(1.82-2.40)
56 h	10.60[d]	8.98[cd]	2.21[d]	1.85[d]

	(9.63-11.61	(7.01-11.16)	(1.96-2.49)	(1.51-2.26)
64 h	9.93[cd]	9.55[d]	2.25[d]	2.01[d]
	(9.31-10.56)	(8.36-10.81)	(2.06-2.45)	(1.81-2.22)
72 h	9.91[cd]	10.83[d]	2.32[d]	2.15[d]
	(9.09-10.78)	(9.63-12.09)	(2.22-2.42)	(1.95-2.37)

[1] Means in columns followed by different superscript letters are significantly different at P=0.05.